Changes in *MYCN* expression in human neuroblastoma cell lines following cisplatin treatment may not be related to *MYCN* copy numbers

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Abstract. Neuroblastoma is a tumor accounting for approximately 10% of all childhood malignancies and 50% of all childhood cancer-related deaths. *MYCN* gene copy number variation represents the most important prognostic factor in neuroblastoma. Prognostic significance of *MYCN* gene expression is more complicated and may depend on other factors such as *MYCN* gene copy number status. In the present study, we assessed *MYCN* gene expression using real-time RT-PCR following cisplatin treatment in three human neuroblastoma cell lines (UKF-NB-3, UKF-NB-4 and SK-N-AS) and their cisplatin-resistant counterparts. We also examined *MYCN* gene status and copy number (gain and amplification) variations using interphase and metaphase fluorescent *in situ* hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA). Only cisplatin-sensitive UKF-NB-4 cells exhibited decreased *MYCN* expression following treatment with cisplatin. Other sensitive neuroblastoma cells did not exhibit a change in *MYCN* expression. In contrast, cisplatin-resistant UKF-NB-4 and SK-N-AS cells exhibited increased *MYCN* expression irrespective of the number of *MYCN* copies or concentration of cisplatin in the medium. In *MYCN*-amplified neuroblastoma cells we did not observe any significant change in the number of *MYCN* copies after cisplatin treatment, whereas *MYCN*-non-amplified SK-N-AS cells revealed during cisplatin treatment an increased number of *MYCN* gene copies caused by 2p gain in the majority of cells by FISH. We postulated that cisplatin treatment does not result directly in altered transcription of *MYCN*. A functional change in *MYCN* mRNA levels and increased *MYCN* expression in cisplatin-resistant neuroblastoma cells do not have a clear relationship to *MYCN* copy numbers. These findings may further contribute to the understanding of cisplatin chemotherapy in connection with *MYCN* expression, and the possible copy number variations in *MYCN* neuroblastoma cells may be of importance since targeting of *MYCN* is being tested as neuroblastoma therapy.

Introduction

Neuroblastoma (NBL), a tumor of the sympathetic nervous system, is the most common solid extracranial childhood tumor, and is responsible for 50% of all childhood cancer-related deaths. V-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived (*MYCN*) was the first amplified oncogene that was found to be of high clinical significance due to its association with the most aggressive form of NBL (1,2). *MYCN* belongs to the family of *MYC* genes (*MYC*, *MYCN* and *MYCL*) which encode transcription regulators that participate in the control of both cell growth and apoptosis depending on the cellular context (3). In healthy humans, *MYCN* is expressed only in certain embryonal tissues, and in adult cells its expression is very low or missing (4). The human *MYCN* gene is normally located on the short arm of chromosome 2 (2p24). The term 2p24 gain is suggested as a descriptive generic term for *MYCN* signal numbers exceeding up to 4-fold the number of reference signals on the chromosomal arm 2q. This pattern could reflect either a gain of short arms of chromosome 2 (2p gain) or a gain of the *MYCN* gene (*MYCN* gain) which is not an independent prognostic factor in NBL (5) and could indicate an 'incipient' *MYCN* amplification (6). *MYCN* amplification is defined as a >4-fold increase in the *MYCN* signal number compared to the reference probe, located on chromosome 2q (6). Amplified *MYCN* can take the form of either extra-chromosomal double minutes or as homogeneously staining chromosomal regions (HSRs), which are usually located on chromosomes other than 2p (normal *MYCN* location) (7).

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Three morphologically distinct cell types termed N (neuroblastic), S (substrate adherent) and I (intermediate) occur in NBL. The biological differences between S and N subtypes may be of clinical relevance (8); S-type cells are often prone to develop drug resistance (9). An effective therapy for children with high-risk neuroblastoma (HR NBL) remains one of the greatest challenges for pediatric oncologists. Over the past two decades, numerous attempts have been made to improve the outcomes of these patients by delivering intensive platinum cytostatics based on induction therapy (10). Unfortunately, intensive chemotherapy, which is usually used for treatment of HR NBL, can cause genetic and expression changes in NBL cells. These cellular changes can cause development of drug resistance to the drugs used, thus, ultimately rendering the chemotherapy ineffective. Drug resistance has been described as common in children suffering from NBL, particularly in those whose tumors display amplification and high-level expression of the *MYCN* oncogene (11). High-level *MYCN* expression is associated with unfavorable outcomes and a poor prognosis in tumors with amplified *MYCN* (12). However, high-level mRNA *MYCN* expression in NBL lacking *MYCN* amplification appears to be associated with favorable outcomes (13). High levels of *MYCN* mRNA in NBL with *MYCN* amplification may be explained by the fact that copies of the amplified gene are actively transcribed (14). Overexpression of the *MYC* gene family generally induces unrestricted cell proliferation, inhibition of differentiation, cell growth, angiogenesis, reduced cell adhesion, metastasis and genomic instability (15). *MYCN* protein has been observed to induce apoptosis in different cells, including NBL and cells with apoptosis induced by cytostatics. NBL cells with *MYCN* amplification (and increased gene expression) can resist treatment only when there is additional dysfunction in the apoptotic pathways, such as caspase deficiency (16). *MYCN* protein can also contribute to the acquired drug-resistance phenotype of cancer cells by modulating the regulation of a specific set of ATP-binding cassette transporter genes to mediate the efflux of chemotherapeutic agents from cancer cells (17).

The aim of our study was to examine *MYCN* gene expression in different NBL cell lines, including cisplatin (CDDP)-sensitive or -resistant N and S types, following a wide range (100-3,000 ng/ml) of different CDDP doses. The novel aspect of the study includes a comparison of *MYCN* gene expression and copies of *MYCN* gene in NBL cells, following long-term CDDP treatment. *MYCN* gene copy numbers and its corresponding expression attracted our particular attention since this gene has at the present become a frequent therapeutic target of NBL (18-20).

Materials and methods

Characteristics of the investigated cell lines. UKF-NB-3 and UKF-NB-4 cell lines with *MYCN* amplification were established from bone marrow metastases of two patients with HR NBL. The UKF-NB-4 cell line that was established from recurrent disease already possessed the intrinsic multidrug resistance phenotype including 7q21 gain (21). The cell lines were kindly provided by Professor J. Cinatl Jr (Institute for Medical Virology, Hospital of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany). Cells were grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). The SK-N-AS cell line was derived from bone marrow metastasis of a female patient with HR NBL. This cell line, which has normal *MYCN* status, was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was cultivated according to the manufacturer's instructions.

The CDDP-resistant counterparts, designated as UKF-NB-3[CDDP] and UKF-NB-4[CDDP], were also kindly provided by Professor J. Cinatl Jr. SK-N-AS[CDDP] was prepared in our laboratory by incubation of parental SK-N-AS with increasing concentrations of CDDP either for 14 or 24 months. Solutions of CDDP (Ebewe Pharma Ges.m.b.H. Nfg. KG, Unterach, Austria) were prepared according to the manufacturer's instructions. CDDP-resistant cell lines were cultivated in a medium containing 1 μg/ml of CDDP.

Subsequent CDDP treatment. The above characterized cells were exposed to 100, 1,000, 2,000 and 3,000 ng/ml CDDP 24 h after seeding. After 24 h, cells were harvested by trypsinization, washed in sterile phosphate-buffered saline and frozen with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at -80˚C until RNA isolation.

MTT test. The sensitivity of cell lines to CDDP was determined by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (22). The IC_{50} was assessed using an MTT dye reduction assay as described in a previous report (23). The IC_{50} values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves using SoftMax Pro microplate data software (Molecular Devices, Sunnyvale, CA, USA).

RNA isolation and real-time reverse transriptase PCR. Total RNA was extracted from all cell lines using TRIzol reagent (Invitrogen). The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, and RNA quantity was measured using a Biomate 3 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Isolated RNA (1 μg) was reverse transcribed to cDNA using random hexamers and a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time RT-PCR was assessed using the 7500 Real-Time PCR System (Applied Biosystems) using *MYCN* and β-2-microglobulin (*B2M*) primers and probe kits from Generi Biotech (Hradec Kralove, Czech Republic) and TaqMan® Gene Expression Master Mix (Applied Biosystems). Results were expressed using the relative expression software tool (REST) (24) employing *B2M* as the reference gene. *B2M* was chosen for its stable expression at consistent levels in NBL, regardless of clinical stage, and have been used as reference genes in a number of studies of gene expression in NBLs or NBL cell lines (25-27).

Fluorescent in situ hybridization and karyotype. Interphase fluorescent *in situ* hybridization (FISH) analyses were performed using an ON *MYCN* (2p24)/LAF (Kreatech Biotechnology B.V., Amsterdam, The Netherlands) and by using Vysis LSI N-MYC (2p24) SpectrumOrange Probe (Vysis, Downers Grove, IL, USA) simultaneously with Alpha

Satellite 2 Green Probe (Cytocell Technologies, Cambridge, UK), in accordance with the manufacturer's instructions. The use of a 2p-specific probe simultaneously with 2q signals is recommended by the International Neuroblastoma Risk Group Biology Committee (6). Slides were scored with an Olympus AX70 (Olympus Optical Co., Ltd., Japan) fluorescence microscope with a CCD camera (Jenoptik, Jena, Germany). Images of the interphase nuclei were digitally captured using an appropriate software program (MetaSystems, Altlussheim, Germany). Two hundred nuclei were investigated from each cell line as recommended for evaluation by FISH analyses (28).

For karyotype analysis, exponentially growing cells cultivated in medium without CDDP were harvested with trypsin, treated with hypotonic solution, and fixed with 3:1 methanol/acetic acid. Slides were banded using standard trypsin-Giemsa banding (GTG-banding) or were used for metaphase FISH analysis performed by Vysis LSI N-MYC (2p24) SpectrumOrange Probe simultaneously with Alpha Satellite 2 Green Probe in accordance with the manufacturer's instructions.

Multiplex ligation-dependent probe amplification. Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to identify whether the region gained or amplified included adjacent genes in addition to the *MYCN* gene. We used the neuroblastoma-specific SALSA MLPA P252B Probe Kit (MRC-Holland, Amsterdam, The Netherlands) which analyzes chromosomes 2 and 17. Detailed information on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com. Genomic DNA (50-200 ng) was denatured and the probes were allowed to hybridize (16 h at 60˚C). PCR was performed on the samples in a volume of 50 μl containing 10 μl of the ligation reaction mixture and using a thermal cycler Mastercycler ep gradient (Eppendorf, Hamburg, Germany). Aliquots of 1 μl of the PCR reaction were combined with 0.5 μl ROX-labeled internal size standard (Applied Biosystems) and 12 μl deionized formamide. Fragments were separated by electrophoresis on an Applied 3130xl capillary sequencer and quantified using the intermediate version Coffalyser (MRC Holland). For copy-number detection, four normal control DNA samples were included in each set of MLPA experiments. Interpretation was based on the comparison of peak heights between the control DNA and the tumor sample.

Statistical analysis. All numerical data are presented as means ± standard deviation and analyzed statistically using the Student's t-test; mRNA expression results were analyzed using REST software (24).

Results

The sensitivity of cell lines to CDDP. The sensitivity of the cell lines to CDDP was verified using the MTT test. The resistant cell lines showed at least a 2-fold increase in resistance to CDDP measured as IC_{50}. The data presented in Table I indicated the UKF-NB-3 cell line as the most sensitive and SK-N-AS^{CDDP} and UKF-NB-4^{CDDP} as the most resistant towards CDDP.

Table I. Levels of the drug-resistance of the tested NBL cell lines expressed as IC_{50}.

Cell line	NBL type	IC_{50} (μM) CDDP
SK-N-AS	S	3.71±0.67
SK-N-AS^{CDDP}	S	11.68±2.84
UKF-NB-3	N	0.99±0.01
UKF-NB-3^{CDDP}	N	7.68±1.37
UKF-NB-4	S	4.60±0.2
UKF-NB-4^{CDDP}	S	11.33±1.12

IC_{50} values were calculated from the linear regression of the dose-log response curves after a 96-h exposure to CDDP, as determined by the MTT assay. Values are the means ± SD of at least 3 independent experiments. S, S-type of NBL; N, N-type of NBL.
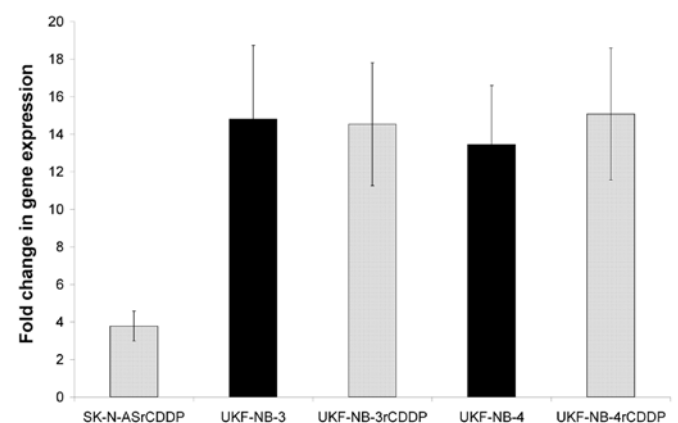

Figure 1. *MYCN* expression in NBL cell lines and their CDDP-resistant (rCDDP) counterparts. The data are expressed as relative expression levels in comparison with the reference NBL cell line SK-N-AS. Expression analysis of reference gene β-2-microglobulin was carried out parallel to all experiments. mRNA levels were not different in any expression analysis for the particular cell line.

MYCN expression levels in CDDP-sensitive and -resistant NBL cell lines. Both *MYCN*-amplified UKF-NB-3 and UKF-NB-4 cell lines had significantly higher *MYCN* mRNA expression as compared to the *MYCN* non-amplified line SK-N-AS (14.8, respectively 13.4-fold higher in 2-log scale, P<0.001) (Fig. 1). SK-N-AS^{CDDP} had 3.8-fold higher expression of *MYCN* (P<0.01) than SK-N-AS. We did not find any distinguishable change in *MYCN* expression between CDDP-sensitive UKF-NB-3 and its CDDP-resistant counterpart (Fig. 1), while UKF-NB-4^{CDDP} had a 1.6-fold higher expression of *MYCN* (P<0.05) in comparison to that in the sensitive UKF-NB-4 cell line. There were no significant differences in *MYCN* expression between UKF-NB-3 and UKF-NB-4 cells nor between UKF-NB-3^{CDDP} and UKF-NB-4^{CDDP} cell lines. Regarding the CDDP-resistant cell lines, the highest *MYCN* expression in comparison with SK-N-AS^{CDDP} was found in the UKF-NB-4^{CDDP} cell line (11.3-fold higher; P<0.01), followed by UKF-NB-3^{CDDP} (10.8-fold higher; P<0.01).

MYCN expression levels in CDDP-sensitive and -resistant NBL cells following short-term CDDP treatment. CDDP-
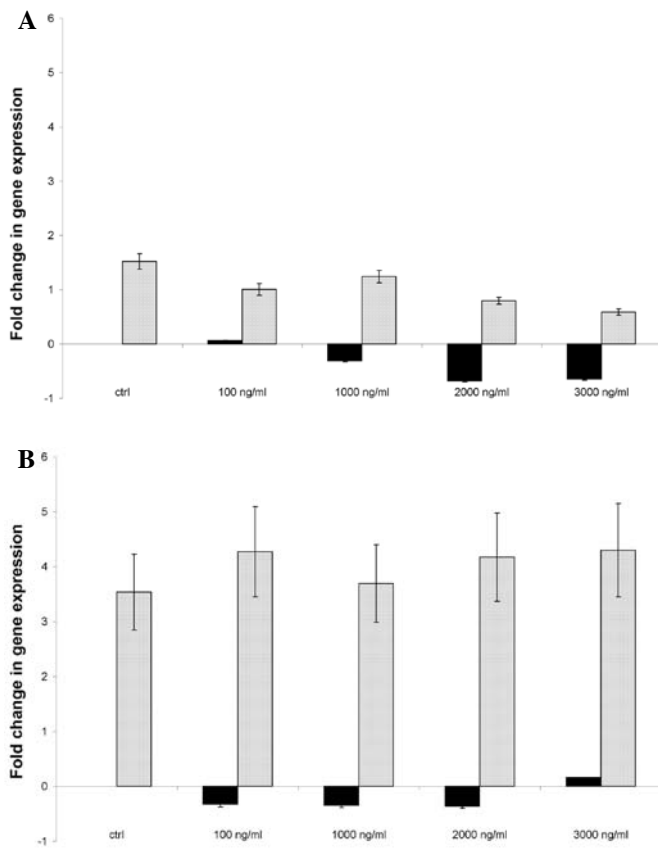
Figure 2. (A) *MYCN* expression in UKF-NB-4 and UKF-NB-4[CDDP] cells after treatment with CDDP. CDDP-sensitive-maternal UKF-NB-4 cells are represented by black bars, CDDP-resistant UKF-NB-4 cells are represented by gray bars. The dose of CDDP is depicted on the x-axis. Untreated CDDP-sensitive UKF-NB-4 cells were used as a reference value. Each experiment was repeated three times with concordant outcomes. (B) *MYCN* expression in SK-N-AS and SK-N-AS[CDDP] cells after treatment with CDDP. CDDP-sensitive-maternal *MYCN* non-amplified SK-N-AS cells are represented by black bars, CDDP-resistant SK-N-AS cells are represented by gray bars. The dose of CDDP is depicted on the x-axis. Untreated CDDP-sensitive SK-N-AS cells were used as a reference value. The experiment was repeated twice.
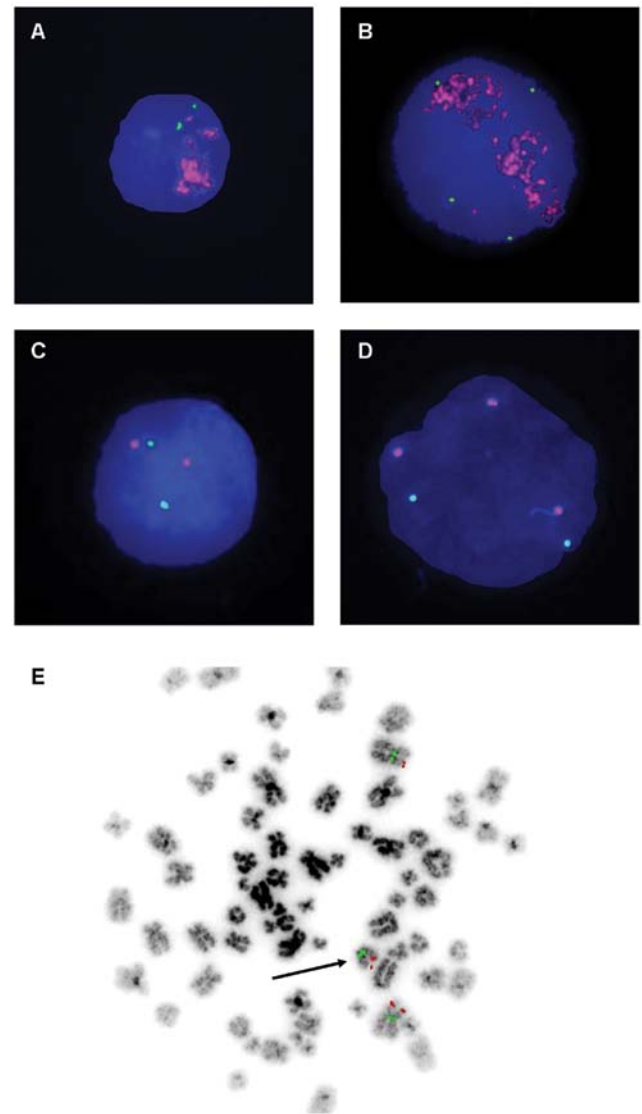

Figure 3. *MYCN* gene in tested NBL cell lines. FISH displays *MYCN* amplification (red signals) in nuclei of (A) UKF-NB-3 and (B) UKF-NB-4 cells. (C) The SK-N-AS cell line has two copies of the *MYCN* gene and two chromosomes 2 (green signals). (D) In the CDDP-resistant SK-N-AS cells, the nuclei exhibited an extra copy of the *MYCN* gene (red signals) representing either 2p gain or *MYCN* gain or both. FISH analyses were performed using ON MYCN (2p24)/LAF probes (original magnification, x100). (E) Mitotic chromosomes of SK-N-AS[CDDP] cells showed an extra copy of the short arm of chromosome 2 (highlighted by the black arrow) containing the *MYCN* gene (red dots) and an extra copy of centromere (green dots) representing 2p gain. FISH was performed using LSI N-MYC (2p24) and chromosome 2 Alpha satellite probes (original magnification, x100).

sensitive UKF-NB-4 cells showed a significant decrease in *MYCN* expression after cultivation with CDDP concentrations of 1000 ng/ml and higher (P=0.001) (Fig. 2A). CDDP-resistant UKF-NB-4 and SK-N-AS cells showed significant upregulation of *MYCN* expression (P=0.001) in comparison with the sensitive cells, however, a single CDDP dose did not significantly modulate *MYCN* expression levels in the resistant cell lines (Fig. 2). These expression changes were not associated with senescence, as determined by staining of investigated cell cultures for the senescence-associated β-galactosidase marker (data not shown).

In the sensitive SK-N-AS cells, we did not observe any significant changes in MYCN expression after cultivation with any CDDP dose (P>0.3). The SK-N-AS[CDDP] cell line showed a significant increase in *MYCN* expression relative to the control maternal line independent of the presence or absence of CDDP in the medium (P<0.05) (Fig. 2B).

MYCN status in CDDP-treated NBL cells. Using FISH, we observed *MYCN* amplification in all 200 investigated nuclei of UKF-NB-3 and UKF-NB-4 cells and their CDDP-resistant counterparts (Fig. 3A and B). UKF-NB-3 and UKF-NB-4

amplified *MYCN* in the form of HSRs. Regarding the UKF-NB-3 cells, multiplex ligation-dependent probe amplification (MLPA) revealed disomy of chromosome 2 without any other genetic aberration in 2p except for *MYCN* amplification. In UKF-NB-3[CDDP] cells we found a loss of the 2p11.2-2p25.3 region except for 2p23.2 (*ALK*) and *MYCN*-amplified copies. Both CDDP-sensitive and -resistant UKF-NB-4 cells exhibited an amplified 2p24.3 region (*NAG*, *DDX1* and *MYCN* genes), but without any other significant change in 2p.

Using interphase FISH analysis with two different sets of probes, the *MYCN*-non-amplified SK-N-AS cell line showed
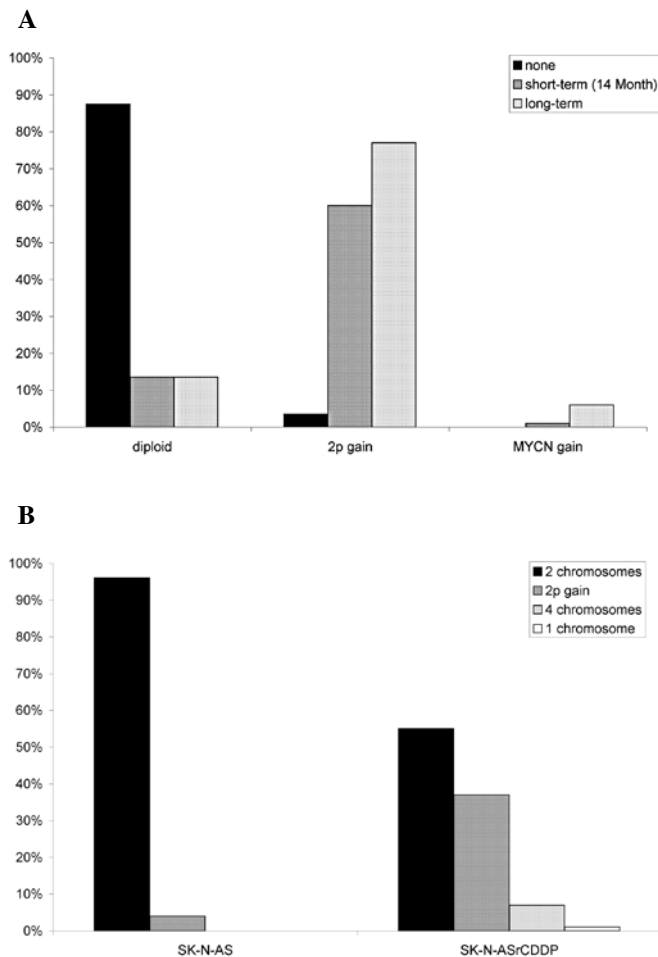
A

B

Figure 4. (A) The number of chromosome 2 and percentage of cells with *MYCN* gain in the SK-N-AS cell lines follows cultivation with CDDP. For the assessment, 200 interphase nuclei of the tested cell line were scored. Three signals of the LSI N-MYC (2p24) in relation to 3 signals of the chromosome 2 Alpha satelite probe were evaluated as 2p gain. More than 3 signals of 2p24 were evaluated as *MYCN* gain. (B) The number of chromosome 2 in mitotic SK-N-AS cells. In each mitosis the number of chromosome 2 was scored and expressed as a percentage of cells with normal and abnormal number of chromosome 2. The type of chromosome 2 gain/loss is also indicated. Investigated cells were prepared by typical cytogenetic preparation using colcemid for metaphase observation. Analyses were performed using LSI N-MYC (2p24) and chromosome 2 Alpha satellite probes.

normal *MYCN* status in the majority of cells (Fig. 3C) with a sporadically (3.5%) occurring clone having an extra 2p (Fig. 4A). After short-term (14 months) and long-term (24 months) cultivation with CDDP, SK-N-AS^CDDP cells were examined again for the *MYCN* gene status and for the number of chromosome 2. The CDDP cultivation resulted in a substantial clonal expansion of cells with 2p gain, since the SK-N-AS^CDDP cells had 60 and 77% of cells with three 2p (Fig. 3D) after short-term and long-term cultivation, respectively (Fig. 4A). In contrast, using MLPA we did not find any significant 2p change in SK-N-AS^CDDP cells, but MLPA analyses whole genomic material regardless of heterogeneity in the cell population as in FISH.

Metaphase FISH analyses of SK-N-AS cells also revealed a predominant occurrence of cells with two chromosomes 2 in mitotic cell and a subsequent increase in cells with 2p gain after CDDP treatment was noted (Fig. 3E). We also observed

cells with tetrasomy or monosomy, but their proportion was rather minor (Fig. 4B).

Discussion

In the present study, 6 NBL cell lines (4 with *MYCN* amplification and 2 cell lines without *MYCN* amplification) were analyzed. The cell line SK-N-AS^CDDP (without *MYCN* amplification), which may serve as a model for the development of drug-resistant NBL, was established and characterized in our laboratory. In the context of CDDP-based NBL therapy, we investigated mRNA *MYCN* expression and *MYCN* gene status. In the SK-N-AS and SK-N-AS^CDDP cells we found a clear link between the number of *MYCN* gene copies and their expression. This finding was in agreement with the study of Valent *et al* (29) who found higher *MYCN* expression in three of four NBLs with 2p/*MYCN* gain in comparison with the SK-N-SH cell line that has 1 *MYCN* copy in the haploid genome. The cells with high-level *MYCN* amplification (UKF-NB-3, UKF-NB-4 and their CDDP-resistant counterparts) exhibit several-fold higher mRNA *MYCN* expression than those without *MYCN* amplification. In the case of NBL cell lines with normal number of *MYCN* copies and in NBL cell lines with one extra *MYCN* copy, the difference in *MYC*N expression was significant. The biological and clinical characteristics of 2p/*MYCN* gain have not been to date as clearly defined as *MYCN* amplification (30). Approximately 10% of diagnosed NBLs could contain an *MYCN* gain (30,31). Patients with *MYCN* gain demonstated a significantly higher age at diagnosis and according to several studies are associated with an advanced stage as compared to *MYCN*-normal cases (30,31). In contrast to the study of Spitz *et al* (31) who reported that NBLs with *MYCN* gain displayed no increase in *MYCN* mRNA expression, and in agreement with Valent *et al* (29) who found in the majority of NBLs cases a 2p/*MYCN* gain increased expression, we found a tendency of *MYCN* gain and higher *MYCN* expression in the SK-N-AS^CDDP cells.

Numeric changes of 2p in SK-N-AS cells may suggest the selection advantage of cells with 2p gain in the presence of CDDP. The simultaneous coexistence of both cell types, cells with disomy of chromosome 2 and cells with 2p gain, is documented in Fig. 4B. The subsequent clonal expansion of cells with 2p gain in the presence of CDDP may be a realistic interpretation of this phenomenon as described by Cohen *et al* (32) in the case of the SK-N-SH cell line. Apparently, senescence has no effect on 2p gain or increased *MYCN* expression in SK-N-AS^CDDP cells. Notably, observations based on metaphase FISH analyses of SK-N-AS^CDDP cells (revealing cells with 2p gain, sporadic tetrasomy or monosomy) have not been confirmed by MLPA analysis, which did not confirm any significant 2p change in both SK-N-AS or SK-N-AS^CDDP cell lines. This discrepancy may be due to the nature of the two techniques: FISH may be more subjective due to the visual evaluation, while capable of taking into account the heterogeneity in the cell population, wherease MLPA, an accurate and sensitive technique, analyzes whole genomic material irrespective of the situation in particular cells (33). Our data suggest the need to analyze amplification of the genetic material by two independent methods in order to avoid false interpretations.

In addition to clinical (clinical stage, age), biochemical (LDH levels) and genetic (*MYCN* amplification, 1p36 deletion) prognostic parameters, the expression of several genes have also been reported to predict the outcome of NBL. Overexpressed genes encoding tyrosine kinase receptors (TrkA and EPHB6) and cell surface molecules (CD44, EFNB2, and EFNB3) have been described as favorable markers (13), but the significance of *MYCN* expression remains controversial since the survival of children suffering from NBL does not correlate with its expression (13,34,35). A high expression of *MYCN* in NBL cells may be associated with both a favorable (cells without *MYCN* amplification) and adverse prognosis (cells with amplified *MYCN*) (12). Similarly, *MYC* overexpression without amplification has also been linked to a more favorable prognosis in patients with breast (36) and colorectal cancers (37). Better outcomes of patients with tumors that overexpress *MYC* or *MYCN* are due to either increased apoptosis or enhanced drug-sensitivity evoked by higher proliferation (35). Clinical observation has also been confirmed in *in vitro* experiments. Transfection of cells with a vector containing *MYCN* significantly decreased the viability of NBL cells without *MYCN* amplification. *MYCN* protein induced apoptosis and enhanced expression of genes that were prognostically favorable (13). *In vitro* studies using an NBL cell line with a controlled *MYCN* expression system showed that NBL cells with induced *MYCN* expression have higher cytostatically induced apoptosis than NBL cells without induction of *MYCN* expression. Cytostatic drugs with various mechanisms of action including CDDP have been tested. All tested compounds were more effective in cells overexpressing *MYCN*, except for betulinic acid, which induces apoptosis by direct effects on mitochondria (38). The above-mentioned results reporting equal or increased *MYCN* expression in CDDP-resistant cell seem to differ from our findings. An explanation may be that the induction of multiple changes in the genome of resistant cells indicates that cytostatics induce drug-resistance through multiple mechanisms (21,39). It is possible that NBL cells without amplification of the *MYCN* used in the above mentioned study (38) have normally functioning apoptotic pathways, but in relation to drug-resistance, there is a defect in one of these pathways, possibly due to increased *MYCN* expression. Van Noesel *et al* (40) analyzed a panel of NBL cell lines with *MYCN* amplification and showed that *MYCN* induces both caspase-8- and caspase-9-mediated apoptosis. The authors concluded that an epigenetic downregulation of caspase-8 in NBLs is unlikely to be induced by overexpression of *MYCN*. Additional defects in apoptosis may be involved in the caspase-9 route to apoptosis. It appears that defects in apoptotic routes in NBL tumors do have a neutralizing effect on *MYCN* (40).

As an *in vitro* model of drug-resistance (22), we chose CDDP-resistant sublines prepared by incubation of parental NBL cells with increasing concentrations of CDDP. We found that the NBL cell line, with predominantly normal status of the *MYCN* gene, had increased numbers of cells with 2p gain and increased *MYCN* expression after CDDP treatment. Wasenius *et al* (41) described both deletion and gain of 2p in CDDP-resistant ovarian cancer cell lines but our finding of 2p gain in NBL in connection with drug-resistance was described for the first time here. We also studied *MYCN* expression in CDDP-sensitive and -resistant NBL cells after exposure to CDDP as an *in vitro* model of chemotherapy. *MYCN* amplified cell lines, cultivated with CDDP, did not change *MYCN* expression. Therefore, cisplatin resistance does not result in a functional change to *MYCN* mRNA levels. Our findings contribute to the understanding of the influence of chemotherapy on *MYCN* expression in NBL cell lines. This is of particular importance since targeting the *MYCN* gene may be a therapeutic alternative for NBL (42).

Acknowledgements

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